HIGH-ACTIVITY PHYTASE COMPOSITIONS

Cross Reference to Related Applications

This application claims the priority of U.S. Provisional Patent Application 60/048,611 filed June 4, 1997, now abandoned, and European Patent Application No. 97201641.4 filed in The Netherlands on June 4, 1997. These applications hereby are incorporated in their entireties by reference.

Field of the Invention

The present invention relates to the preparation and formulation of phytase enzymes and their use to prepare granulates for feed-enzymes in animal feeds.

Background of the Invention

The use of various enzymes such as phytases in animal, e.g., livestock, feed is becoming more common. These enzymes are included in order to improve nutrient or mineral uptake from the feed by the animal, and may also help digestibility. They are usually produced by culturing microorganisms in large scale fermenters operated by industrial enzyme producers. At the end of the fermentation the resulting "broth" is usually subjected to a series of filtration steps to separate the biomass (the microorganisms) from the desired enzyme (in solution). The enzyme solution is either then sold as a liquid (often after addition of various stabilizers) or processed to a dry formulation.

Enzyme liquid and dry formulations are used on a commercial scale by the animal feed industry. Liquid formulations may be added to the feed after pelleting in order to avoid heat inactivation of the enzyme which would occur during the pelleting process.

Dry formulations usually involve steam pelleting where the feed is subjected to steam injection(s) prior to pelleting. In the subsequent pelleting step the feed is forced through a matrix or die and the resulting strips are cut into suitable pellets of variable length. During this process temperatures may rise to 60-95°C.

Phytases are enzymes that (at least partially) hydrolyse phytate (*myo*-inositolhexakis phosphate) to *myo*-inositol and inorganic phosphate. These enzymes are found in wheat bran, plant seeds, animal intestines and can be produced by

microorganisms. Phytases are provided in animal feeds because, as they are able to degrade phytate, they can increase the availability of phosphorus and other nutritional components to the animal. Phytases can also increase the digestibility of calcium.

Phosphorus is an essential element for the growth of organisms. For livestock, the feed is often supplemented with inorganic phosphorus in order to obtain good growth in monogastric animals. There is however often no need for this in feedstuffs of ruminants because microorganisms present in the rumen produce enzymes that catalyse the conversion of phytate to inositol and inorganic phosphate. The degration of phytate is often desirable because phytic acid can be anti-nutritional as it chelates useful minerals such as calcium, zinc, magnesium and iron, and can also react adversely with proteins thereby decreasing their bioavailability to the animal. The addition of phytase may also reduce the amount of inorganic feed that needs to be added, and so less phosphorus is excreted in the manure which is better for the environment.

The gene for various phytase enzymes have been cloned and expressed. EP-A-0,420,358 (Gist-Brocades) describes the expression of microbial phytases.

In a later application EP-A-0,684,313 (Hoffmann-La Roche) describes a DNA sequence coding for various polynucleotides having phytase activity.

EP-A-0,758,018 (Gist-Brocades) refers to methods of improving the stability of enzymes, especially for use as animal feeds, and refers to phytases.

WO-A-94/03612 (Alko) describes the production of phytase degrading enzymes in *Trichoderma* while WO-A-97/16076 (Novo Nordisk) describes enzyme-containing preparations for use in the manufacture of animal feeds comprising various hydrophobic substances.

Animal feed represents one of the largest costs incurred in keeping livestock and other animals. Furthermore, additives such as enzymes like phytase can add significantly to the cost of animal feed. One aim of the present invention is to be able to provide phytase compositions that are cheaper to produce. This can be achieved by being able to manufacture high activity or highly concentrated phytase compositions, as provided by the present invention.

An additional advantage in being able to make high activity phytase compositions as provided by the present invention is that these compositions can show a marked increase in stability, especially during a pelleting process in the

preparation of animal feed (pellets), and so are more likely to retain the higher phytase activity than the compositions of the prior art over time. Further objects and advantages of the invention will be apparent from the description herein.

Brief Summary of the Invention

The present invention provides high-activity phytase compositions and methods for the preparation of same. The compositions can be employed *inter alia* in the preparation of granulates that can be incorporated into animal feeds.

Detailed Description of the Invention

In a first preferred aspect of the present invention there is provided a process for the preparation of an aqueous liquid comprising a phytase, the process preferably comprising:

(a) culturing in an aqueous medium a microorganism of the genus *Aspergillus or Trichoderma* having a heterologous (i.e., foreign to the host) phytase gene under the control of a glucoamylase (for *Aspergillus)* or cellobiohydrolase (for *Trichoderma*) promoter, under conditions that allow recombinant expression of the phytase, where the medium comprises, as a feed for the microorganism, an assimilable carbon source and an assimilable nitrogen source;

(b) filtering the aqueous medium to remove the microorganisms to give an aqueous filtrate; and

(c) subjecting the filtrate from (b) to ultrafiltration to give an aqueous liquid having a phytase concentration of at least about 14,000 FTU/g.

This process has been found to provide a particularly high concentration of phytase in the resulting aqueous composition. This has allowed the preparation of other phytase compositions, also at high activity levels, which means that not only is the process cheaper (per unit of enzyme activity), but also, the more concentrated phytase-containing compositions have been found to be much more stable than their less concentrated counterparts.

The microorganisms are preferably of the species *Aspergillus niger, Aspergillus oryzae* or *Trichoderma reesei.* For the *Aspergillus* organisms, the phytase gene is suitably under the control of a glucoamylase (or amyloglucosidase, AG) promoter. For *Trichoderma* organisms, it is preferred to use a cellobiohydrolase

promoter. Desirably the promoter is one which is capable of commanding gene expression in these respective hosts.

The assimilable carbon source can comprise glucose and/or maltodextrin, and/or the assimilable nitrogen source can comprise ammonium ions. The glucose and ammonium ions can be the only assimilable carbon or nitrogen sources in the aqueous medium. That is to say, it is contemplated that no complex carbon or nitrogen sources are used. The ammonium ions can be provided either as ammonia or an ammonium salt. Preferred ammonium salts include ammonium nitrate, ammonium sulphate and ammonium phosphate.

Preferably the carbon and/or nitrogen source is supplied to the culture medium during the fermentation process. The rate of supply of either source can be substantially the same as it is consumed by the microorganisms. Thus the carbon and/or nitrogen source can be provided in a continuous or continual manner. The carbon and nitrogen sources can be provided separately, or in the same supply.

The resulting aqueous liquid can have a phytase concentration of at least about 16,000, and possibly even about 18,000 or more, FTU/g. In particular, the resulting aqueous liquid desirably comprises a phytase concentration of from about 15,000 to about 20,000 FTU/g, and more preferably comprises a phytase concentration of from about 16,000 to about 18,000 FTU/g. One FTU (phytase unit) is the amount of enzyme which liberates 1 micromole of inorganic phosphorus per minute from 0.0051 Mol/L of sodium-phytate at pH 5.5 and 37°C. FTU can be calculated using any standard means, and, desirably, using the method of Engelen et al., Journal of AOAC International, 77 (3), 760-764 ("Simple and Rapid Determination of Phytase Activity"), and more particularly, as set forth in Example 2. Further, phytase activity can be determined according to the procedure "ISL-method 61696 (manual vanadate assay) which is obtainable on request from Gist-brocades, Food Specialties, Agri Ingredients Group, Wateringseweg 1, P.O. Box 1, 2600 MA, Delft, The Netherlands.

By using these particular organisms under these conditions the filtrate will be relatively concentrated. This allows it to be subjected to ultra-filtration. In some prior art methods, the resulting filtrate contains too much debris and other substances to allow ultra-filtration (the filter clogs). However, in the process of the present invention, the filtrate is relatively "clean" which allows the filtrate to be subjected to ultra-filtration, without any further processing, and by ultra-filtration a particularly

high concentrated aqueous composition can therefore be obtained.

Prior art methods have discussed the possibility of subjecting either the filtrate or the aqueous composition to either crystallization and/or colour removal steps, for example charcoal filtration. However, both of these additional steps (which would add to the cost of producing the phytase) can be dispensed with in the present invention.

Preferably the microorganisms do not possess, or at least do not express, a glucoamylase gene. This means that the microorganism can devote more energy to the production of the phytase.

The microorganism can possess multiple copies of the phytase gene. This has been found to increase the production levels of the phytase because there are more phytase genes to be expressed.

The aqueous composition can be substantially free of taka-amylase (i.e., a well-known α-amylase such as described in Toda et al., Proc. Japan Acad., 58 Ser. B 208-212 (1982)).

In the process of the invention it is preferred that (substantially) all of the carbon and/or nitrogen sources have been consumed by the microorganisms before the filtering in (b) takes place. This can be achieved by allowing fermentation to continue for some time after the last supply of carbon and/or nitrogen sources have taken place. Alternatively, one can allow the fermentation to continue beyond the stage when all of the carbon and/or nitrogen sources have been added. The advantage of this, as will be apparent, is that the aqueous composition can then be (substantially) free of the carbon and/or nitrogen sources (e.g., the glucose and/or ammonium ions). Once again, this can make for a cleaner aqueous liquid, which can contain fewer by-products. By reducing the number of by-products one can minimise the number of processing steps required to be able to either use the aqueous liquid, or to be able to obtain a desired high phytase activity.

The most preferred organism is *Aspergillus niger*. Also preferred is that the phytase is expressed in a microorganism with a glucoamylase signal sequence.

The resulting aqueous phytase-containing liquid can then be used for a variety of purposes, although its application in animal feeds is specifically contemplated here. A second aspect of the invention relates to this aqueous liquid, such as preparable by a process of the first aspect, comprising phytase at a concentration of at least about

14,000 FTU/g, desirably from about 15,000 to about 20,000 FTU/g, and even more preferably from about 16,000 to about 18,000 FTU/g.

In the specification a "phytase" means not only naturally occurring phytase enzymes, but any enzyme (i.e., produced by recombinant, synthetic and any other means) that possesses phytase activity, for example, the ability to catalyse the reaction involving the removal or liberation of inorganic phosphorous (phosphate) from *myo*-inositol phosphates. Preferably the phytase will belong to the class EC 3.1.3.8. The phytase itself is preferably a fungal phytase, such as derived from an *Aspergillus* or *Trichoderma* species.

The invention can also provide processes for the preparation of phytase formulations in the form of granulates that use an edible carbohydrate polymer as a carrier. The carrier may be in particulate or powder form. The phytase-containing aqueous liquid, such as a solution or a slurry, can be mixed with the solid carrier and allowed to absorb onto the carrier. During or after the mixing, the phytase-containing liquid and the carrier are processed into a granulate, which can then subsequently be dried. The use of the carbohydrate carrier may allow the absorption of large amounts of the composition (and therefore phytase). The mixture can be used to form a plastic paste or non-elastic dough that can readily be processed into granules, for example it is extrudable. Suitably the carrier is non-fibrous which allows for easier granulation: fibrous materials can prevent granulation by extrusion.

A number of prior art documents refer to pellets containing various enzymes, but these find use as detergents, often in washing compositions. In contrast, the present application finds use in animal feeds and for that reason the granulates of the invention are edible (by animals) and preferably also digestible. It therefore is a surprising and unexpected aspect of the present invention that the granulates, granules, and compositions of the invention are free of soap, detergents and bleach or bleaching compounds, zeolites, binders, fillers (TiO_2, kaolin, silicates, talc, etc.) to name but a few.

The edible carbohydrate polymer should be chosen so that it is edible by the animal for whom the feed is intended, and preferably digestible as well. The polymer preferably comprises glucose (e.g., a glucose-containing polymer), or $(C_6H_{10}O_5)_n$, units. Preferably the carbohydrate polymer comprises α-D-glucopyranose units, amylose (a linear (1→4) α-D-glucan polymer) and/or amylopectin (a branched D-

glucan with α-D-(1→4) and α-D-(1→6) linkages). Starch is the preferred carbohydrate polymer. Other suitable glucose-containing polymers that can be used instead of, or in addition to starch, include α-glucans, β-glucans, pectin (such as proto-pectin), and glycogen. Derivatives of these carbohydrate polymers, such as ethers and/or esters, thereof are also contemplated, although gelatinised starch is often avoided. Suitably the carbohydrate polymer is water-insoluble.

In the examples described herein corn-, potato- and rice-starch is used. However, starch obtained from other (e.g., plant, such as vegetable or crop) sources such as tapioca, cassava, wheat, maize, sago, rye, oat, barley, yam, sorghum, or arrowroot is equally applicable. Similarly both native or modified (e.g., dextrin) types of starch can be used in the invention. Preferably the carbohydrate (e.g., starch) contains little or no protein, e.g., less than about 5% (w/w), such as less than about 2% (w/w) preferably less than about 1% (w/w). Even more desirably the carbohydrate contains from about 0.005% (w/w) to about 1% (w/w).

At least about 15% (w/w) of the solid carrier can comprise the carbohydrate polymer (such as starch). Preferably, however, at least about 30% (w/w) of the solid carrier comprises the carbohydrate, optimally at least about 40% (w/w). Advantageously the major component of the solid carrier is the carbohydrate (e.g., starch), for example more than about 50% (w/w), preferably at least about 60% (w/w), suitably at least about 70% (w/w), and optimally at least about 80% (w/w). Thus, desirably the carbohydrate comprises from about 40% to about 100% (w/w), particularly from about 70% to about 90% (w/w) of the solid carrier. These weight percentages are based on the total weight of the non-enzymatic components in the final dry granulate.

The amount of phytase-containing liquid that can be absorbed onto the carrier is usually limited by the amount of water that can be absorbed. For natural, granular, starch this can vary between from about 25% (w/w) to about 30% (w/w), without using elevated temperatures (that cause the starch to swell). In practice the percentage of enzyme liquid to be added to the carbohydrate will often be much larger than this because the enzyme containing liquid will usually contain a significant amount of solids. The phytase solution can contain about 25% (w/w) solids (optimally from about 15% to about 40% (w/w), as a result of which the carbohydrate (e.g., starch) and phytase solution can be mixed at a ratio of carbohydrate:phytase solution of from

about 0.5:1 to about 2:1, e.g., from about 1.2:1 to about 1.6:1, such as at a ratio of about 60% (w/w):40% (w/w), respectively. Preferably the amount of liquid added to the solid carrier is such that (substantially) all the water in the (aqueous) liquid is absorbed by the carbohydrate present in the solid carrier.

At elevated temperatures starch and other carbohydrate polymers can absorb much larger amounts of water under swelling. For this reason the carbohydrate polymer is desirably able to absorb water (or enzyme-containing aqueous liquids). For example, corn starch can absorb up to three times its weight of water at 60°C and up to ten times at 70°C. The use of higher temperatures in order to absorb a greater amount enzyme-containing liquid is thus contemplated by the present invention, and indeed is preferable especially when dealing with thermostable phytase enzymes. For these enzymes therefore the mixing of the solid carrier and liquid can be conducted at elevated temperatures (e.g., above ambient temperature), such as above about 30°C, preferably above about 40°C, and optimally above about 50°C. In particular, depending on the thermostability of the enzymes employed, desirably mixing can be done at temperatures from about 30°C to about 80°C. Alternatively, or in addition, the liquid may be provided at this temperature.

However, in general, non-swelling conditions at lower (e.g., ambient) temperatures are preferred to minimise activity loss arising from instability of (heat sensitive) phytases at higher temperatures. Suitably the temperature during the mixing of the enzyme and the liquid is from about 20°C to about 35°C, and more preferably from about 20°C to about 25°C.

The mechanical processing used in the present invention for making the mixture of the phytase-containing liquid and the solid carrier into granules (in other words granulating) can employ known techniques frequently used in food, feed and enzyme formulation processes. This can comprise expansion, extrusion, spheronisation, pelleting, high shear granulation, drum granulation, fluid bed agglomeration or a combination thereof. These processes are usually characterised by an input of mechanical energy, such as the drive of a screw, the rotation of a mixing mechanism, the pressure of a rolling mechanism of a pelleting apparatus, the movement of particles by a rotating bottom plate of a fluid bed agglomerator or the movement of the particles by a gas stream, or a combination thereof. These processes

allow the solid carrier (e.g., in the form of a powder), to be mixed with the phytase-containing liquid (an aqueous solution or slurry), and so subsequently granulated.

In yet a further preferred embodiment of the invention the granulate (e.g., an agglomerate) is formed by spraying or coating the phytase-containing liquid onto the carrier, such as in a fluid bed agglomerator. Here the resulting granules can include an agglomerate as can be produced in a fluid bed agglomerator.

Preferably the mixing of the phytase-containing liquid and the solid carrier additionally comprises kneading of the mixture. This can improve the plasticity of the mixture in order to facilitate granulation (e.g., extrusion).

If the granulate is formed by extrusion this is preferably performed at low pressure. This can offer the advantage that the temperature of the mixture being extruded will not increase, or increases only slightly. Low-pressure extrusion includes extrusion for example in a Fuji Paudal basket- or dome- extruder. Preferably extrusion does not result in the temperature of the material being extruded to rise above about 40°C. The extrusion can naturally produce granules (the granules can break off after passage through a die) or a cutter can be employed.

Suitably the granules will have a water content of from about 30% to about 40%, such as from about 33% to about 37%. The enzyme content is preferably from about 3% to about 10%, e.g., from about 5% to about 9%.

The granules obtained desirably can be subjected to rounding off (e.g., spheronisation) such as in a spheromiser, e.g., a MARUMERISER™ machine and/or compaction. The granules preferably can be spheronised prior to drying since this can reduce dust formation in the final granulate and/or can facilitate any coating of the granulate.

The granules preferably can then be dried, such as in a fluid bed drier or, in case of the fluid bed agglomeration, desirably can be immediately dried (in the agglomerator) to obtain (solid dry) granulates. Other known methods for drying granules in the food, feed or enzyme industry can be used by the skilled person. Suitably the granulate is flowable.

The drying preferably takes place at a temperature of from about 25°C to about 60°C, such as from about 30°C to about 50°C. Here the drying may last from about 10 minutes to about several hours, such as from about 15 minutes to about 10 hours, preferably from about 15 minutes to about 3 hours, or desirably from about 15

minutes to about 30 minutes. The length of time required will of course depend on the amount of granules to be dried, but as a guide this is from about 1 to about 2 seconds per kg of granules.

After drying the granules, the resulting granulate preferably has a water content of from about 3% to about 10%, such as from about 5% to about 9%.

A coating preferably can be applied to the granulate to give additional (e.g., flavoured) characteristics or properties, like low dust content, colour, protection of the enzyme from the surrounding environment, different enzyme activities in one granulate, or a combination thereof. The granules can be coated with a fat, wax, polymer, salt, unguent and/or ointment or a coating (e.g., liquid) containing a (second) enzyme, or a combination thereof. It will be apparent that if desired several layers of (different) coatings can be applied. To apply the coating(s) onto the granulates a number of known methods are available which include the use of a fluidised bed, a high shear granulator, a mixer granulator, or a Nauta-mixer.

In other embodiments additional ingredients can be incorporated into the granulate where desirable, e.g., as processing aids, for further improvement of the pelleting stability and/or the storage stability of the granulate. A number of such preferred additives are discussed below.

Salts preferably can be included in the granulate (e.g., with the solid carrier or liquid). Preferably (as suggested in EP-A-0,758,018) inorganic salt(s) can be added, which can improve the processing and storage stability of the dry phytase preparation. Preferred inorganic salts comprise a divalent cation, such as zinc, magnesium, and calcium. Sulphate is the most favoured anion.

Preferably (as suggested in EP-A-0,758,018) inorganic salt(s) can be added, which can improve the processing and storage stability of the dry enzyme preparation. Preferred inorganic salts are water soluble. They can comprise a divalent cation, such as zinc (in particular), magnesium, and calcium. Sulphate is the most favoured anion although other anions resulting in water solubility can be used. The salts can be added (e.g., to the mixture) in solid form. However, the salt(s) can be dissolved in the water or enzyme-containing liquid prior to mixing with the solid carrier. Suitably the salt is provided at an amount that is at least about 15% (w/w based on the enzyme), such as at least about 30%. However, it can be as high as at least about 60%, or even about 70%, for a maximum range of from about 15% to

about 70% (again, w/w based on the enzyme). These amounts can apply either to the granules or to the granulate. The granulate therefore can comprise less than about 12% (w/w) of the salt, for example from about 2.5% to about 7.5% (w/w), e.g., from about 4% to about 6% (w/w).

If the salt is provided in the water then it optimally can be in an amount of from about 5% to about 30% (w/w), such as from about 15% to about 25% (w/w).

Further improvement of the pelleting stability can be obtained where desirable by the incorporation of hydrophobic, gel-forming or slow dissolving (e.g., in water) compounds. These can be provided at from about 1% to about 10%, such as from about 2% to about 8%, and preferably from about 4% to about 6% by weight (based on the weight of water and solid carrier ingredients). Suitable substances include but are not limited to derivatised celluloses, such as HPMC (hydroxy-propyl-methyl-cellulose), CMC (carboxy-methyl-cellulose), HEC (hydroxy-ethyl-cellulose); polyvinyl alcohols (PVA); and/or edible oils. Edible oils, such as soy oil or canola oil or other appropriate oil, can be added (e.g., to the mixture to be granulated) as a processing aid, although as a rule hydrophobic substances (e.g., palm oil) are preferably absent.

Preferably the granules have a relatively narrow size distribution (e.g., they are monodisperse). This can facilitate a homogeneous distribution of the phytase in the granules and/or the enzyme granulate in the animal feed. The process of the invention tends to produce granulates with a narrow size distribution. However, if necessary, an additional step can be included in the process to further narrow the size distribution of the granules, such as screening. The size distribution of the granulate is suitably between about 100 μm and about 2000 μm, preferably between about 200 μm and about 1800 μm, and optimally between about 300 μm and about 1600 μm. The granules can be of irregular (but preferably are regular) shape, for example, approximately spherical.

Other suitable enzyme(s) can be included in the animal feed which includes pet food. The function of these enzymes is often to improve the feed conversion rate, e.g., by reducing the viscosity or by reducing the anti-nutritional effect of certain feed compounds. Feed enzymes can also be used where desirable, such as to reduce the amount of compounds which are harmful to the environment in the manure. Preferred enzymes for these purposes include but are not limited to: carbohydrases, such as

amylolytic enzymes and plant cell wall degrading enzymes which include cellulases such as β-glucanases, hemicelluloses such as xylanases, or galactanases; peptidases, galactosidases, pectinases, esterases; proteases, preferably with a neutral and/or acidic pH optimum; and lipases, preferably phospholipases such as the mammalian pancreatic phospholipases A2. Preferably, the enzyme does not include starch degrading enzymes (for example amylases). In some embodiments proteases desirably can be excluded as these may cause harm if ingested. If the enzyme is a plant cell wall degrading enzyme, for example a cellulase, and in particular a hemicellulose such as xylanase, then the final granulate desirably can have an activity of the enzyme ranging from about 3,000 to about 100,000, preferably from about 5,000 to about 80,000, and optimally from about 8,000 to about 70,000, EXU/g. If the enzyme is a cellulase, such as β-gluconase, then the final granulate preferably can have an enzyme activity of from about 500 to about 15,000, preferably from about 1,000 to about 10,000, and optimally from about 1,500 to about 7,000, BGU/g.

One EXU (endoxylanase unit) is the amount of enzyme which liberates 1 micromole of reducing sugars (measured as xylose equivalents) per minute from a 1% xylan solution at pH 3.5 and 40°C. One BGU (Betaglucanase unit) is the amount of enzyme which liberates 0.278 micromole reducing sugars (measured as glucose equivalents) per minute at pH 3.5 and 40°C at a substrate concentration of 0.5% betaglucan from barley. EXU and BGU can be calculated using any standard means, and desirably, using the method of Engelen et al., Journal of AOAC International, 79 (5), "Viscosimetric determination of β-glucanase and endoxylanase activity in feed" (1996). Furthermore, EXU and BGU can be calculated, respectively, according to the procedures "ISL-method 62170" (manual viscosimetric assay) and "ISL-method 62169" (manual viscosimetric assay), both protocols of which are obtainable on request for Gist-brocades (Delft, The Netherlands).

The granules desirably can comprise from about 5% to about 20%, e.g., from about 7% to about 15% of the enzyme(s). The enzyme can be naturally occurring or recombinant (i.e., including synthetic).

A preferred process according to the invention therefore comprises:

a. mixing the aqueous phytase-containing liquid and solid carrier comprising at least about 15% (w/w) or an edible carbohydrate polymer, for example mixing the solid carrier with an aqueous enzyme-containing liquid;

b. optionally kneading the resulting mixture;

c. granulating, for example by mechanical processing, the mixture in order to obtain enzyme-containing granules, for example by using a granulator or by extrusion;

d. optionally spheronising the granules;

e. drying the resultant granules to obtain an enzyme-containing granulate.

During the entire process one will aim to keep the maximum temperature to which the enzyme(s) are exposed to below about 80°C. In particular, desirably mixing can be done at temperatures of from about 30°C to about 80°C, and drying can be done at temperatures of from about 25°C to about 60°C.

The granulates of the invention are suitable for use in the preparation of an animal feed. At perhaps its broadest, this aspect of the invention covers a granulate comprising a phytase and an edible carbohydrate polymer, the granulate having an activity of at least about 6,000 FTU/g. In particular, preferably the final granulate has an activity of from about 5,000 to about 10,000 FTU/g, such as from about 6,000 to about 8,000 FTU/g. In such processes, the granulates desirably are mixed with feed substances, either as such, or as part of a premix. The characteristics of the granulates according to the invention allows their use as a component of a mixture which is well suited as an animal feed, especially if the mixture is steam treated and subsequently pelleted. The dried granules desirably can be visible or distinguishable in such pellets.

Thus a third preferred aspect of the present invention relates to a process for the preparation of animal feed, or a premix or precursor to an animal feed, the process preferably comprising mixing a composition of the second aspect with one or more animal feed substances (e.g., seeds) or ingredients. This desirably can then be sterilised, e.g., subjected to heat treatment. The resulting composition is then suitably processed into pellets.

A fourth preferred aspect of the invention relates to a composition comprising a granulate of the second preferred aspect, which is preferably an edible feed composition such as an animal feed. This composition is preferably in the form of pellets (there may be from about 1 to about 5, e.g., from about 2 to about 4, dried granules per pellet).

Suitably the composition comprises from about 0.05 to about 2.0, such as from about 0.3 to about 1.0, optimally from about 0.4 to about 0.6 FTU/g of the phytase. A xylanase can be present at from about 0.5 to about 50 EXU/g, e.g., from about 1 to about 40 EXU/g. Alternatively, or in addition, a cellulase can be present at from about 0.1 to about 1.0, e.g., from about 0.2 to about 0.4 BGU/g.

The composition desirably can have a water content of from about 10% to about 20%, e.g., from about 12% to about 15%. The amount of enzyme(s) is suitably from about 0.0005% to about 0.0012%, such as at least about 5ppm.

A fifth preferred aspect relates to a process for promoting the growth of an animal, the process comprising feeding an animal with a diet that comprises a composition of the second aspect or a composition of the fourth aspect. Here, the animal diet can include either the granulate itself, or the granulate present in a feed.

A sixth preferred aspect of the present invention relates to the use of compositions in, or as a component of, an animal feed or for use in an animal diet.

A seventh preferred aspect of the present invention also relates to the use of a composition comprising at least about 15% (w/w) of an edible carbohydrate polymer as a carrier for a phytase to improve the pelleting stability of the phytase.

Suitable animals include farm animals (e.g., pigs, poultry, livestock and the like), non-ruminants or monogastric animals (e.g., pigs, fowl, poultry, marine animals such as fish and the like), ruminants (e.g., bovine or ovine, such as cows, sheep, goats, deer, calves, lambs and the like). Poultry includes but is not limited to chickens, hens and turkeys.

Preferred features and characteristics of one aspect of the invention are equally applicable to another *mutatis mutandis*.

The following Examples are presented merely to illustrate the invention, and are not intended, or to be construed as, being limiting.

EXAMPLES

EXAMPLE 1

Fermentation of A. niger CBS 513.88

Aspergillus niger fungal spore preparations were made following standard techniques.

Spores and subsequently cells were transferred through a series of batch fermentations in Erlenmeyer flasks to a 101 fermenter. After growth in batch culture, the contents of this fermenter were used as inoculum for a final 500 litre batch fermentation.

The media used contains: 9lg/l corn starch (BDH chemicals); ammonium 38g/l glucose.H_2O; 0.6g/l $MgSO_4$. $7H_2O$; 0.6g/l KC1; 0.2g/l $FeSO_4.7H_2O$ and 12g/l KNO_3. The pH was maintained at 4.6 ± 0.3 by automatic titration with either 4N NaOH or 4N H_2SO_4.

Cells were grown at 28°C at an automatically controlled dissolved oxygen concentration at 25% air saturation. Phytase production reached a maximum level of 5-10 U/ml after 10 days of fermentation.

The fermentation was repeated using ammonium sulphate in place of corn starch (to give an equivalent assimilable nitrogen content).

EXAMPLE 2

Purification and characterization of phytase: phytase activity assay

100µl of broth filtrate (diluted when necessary) or supernatant or 100µl of demiwater as reference are added to an incubation mixture having the following composition:

- 0.25M sodium acetate buffer pH 5.5, or
- glycine HC1-buffer; pH 2.5
- lmM phytic acid, sodium salt
- demiwater up to 900µl

The resulting mixture was incubated for 30 minutes at 37°C. The reaction was stopped by the addition of 1 ml of 10% TCA (trichloroacetic acid). After the reaction had terminated, 2 ml of reagent (3.66g of $FeSO_4.7H_20$ in 50ml of ammonium molybdate solution (2.5g $(NH_4)_6Mo_7O_{24}.4H_2O$ and 8ml of H_2SO_4 , diluted up to 250ml with demiwater) was added.

The intensity of the blue colour was measured spectro-photometrically at 750nm. The measurements are 15 indicative of the quantity of phosphate released in relation to a calibration curve of phosphate in the range of 0-1 mMol/l.

EXAMPLE 3

A. Phytase expression in A. niger CBS 513.86 transformed with expression vectors containing the A. Ficuum phytase gene fused to the promoter and/or signal sequences of the A. niger amyloglucosidase (AG) gene

To obtain overexpression of phytase in *A. niger* an expression cassette was derived in which the A. *ficuum* phytase gene was under control of the A. *niger* amyloglucosidase (AG) promoter in combination a signal sequence. For the longer leader sequence the AG promoter sequence was fused to the phytase encoding sequence including the phytase leader sequence which was fused to the phytase gene fragment encoding the mature protein (see for reference Example 10 of EP-A-0,420,358).

B. Expression of the phytase gene under the control of the AG promoter in A. niger

The A. *niger* strain CBS 513.88 (deposited 10 October 1988 with Centraal bureau Voor Schimmelcultures (CBS), Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, - Netherlands) was transformed with 10μg DNA fragment by known procedures (e.g., see Example 9 of EP-A-0,420,358). Single *A. niger* transformants from each expression cassette were isolated, and spores were streaked on selective acetamide-agar plates. Spores of each transformant were collected from cells grown for 3 days at 37°C on 0.4% potato-dextrose (Oxoid, England) agar plates. Phytase production was tested in shake flasks under the following conditions:

Approximately 1 x 10^8 spores were inoculated in 100ml pre-culture medium containing (per litre) : lg KH_2PO_4; 30g maltose; 5g yeast extract; l0g casein-hydrolysate; 0.5g $MgSO_4.7H_2O$ and 3g Tween 80. The pH was adjusted to 5.5.

After growing overnight at 34°C in a rotary shaker, 1ml of the growing culture was inoculated in a 100ml main-culture containing (per litre): 2g KH_2P0_4; 70g maltodextrin (maldex MDO_3, Amylum); 12.5g yeast extract; 25g casein-hydrolysate; 2g K_2SO_4; 5g $MgSO_4.7H_20$; 0.03g $ZnCl_2$; 0.02g $CaCl_2$; 0.05 $MnSO_4.4H_2O$ and $FeSO_4$. The pH was adjusted to 5.6.

The mycelium was grown for at least 140 hours. Phytase production was measured as described in Example 2. The fermentation was repeated using equivalent amounts of glucose and ammonium sulphate as the carbon and nitrogen sources. The broth was filtered to give a filtrate which was separated from the biomass. Using the expression cassette PFYT3 (AG-promoter/phytase leader) a maximum phytase activity of 280 U/ml was obtained.

EXAMPLE 4

Purification of Phytase from filtrate

The purification to obtain highly purified phytase was as follows:

1. Cation exchange chromatography at pH 4.9
2. Cation exchange chromatography at pH 3.8
3. Anion exchange chromatography at pH 6.3
4. Ultrafiltration

1. The phytase filtrate was diluted 20 times with water and the pH was set at 4.9. This material was passed through a S Sepharose Fast Flow column equilibrated with a 20mM citric acid/NaOH pH 4.9 buffer. The unbound material, with phytase, was collected and used for the next step.

2. The pH 4.9 material was brought to pH 3.8 and the phytase was bound on a S Sepharose Fast Flow column equilibrated with 2-mM citric acid/NaOH pH 3.8 buffer. The phytase was eluted from the column with a 20mM $NaPO_4$, 5OmM NaCl pH 7.6 buffer.

3. The pooled phytase fractions from the second cation exchange step were pH adjusted to 6.3 and the phytase was bound onto a Q Sepharose Fast Flow column equilibrated with a l0mM KPO_4 pH 6.3 buffer. The phytase was eluted using a gradient to 1M NaCl in the same buffer.

Table 1: Summary Purification Results

Summary Purification Results	
Sample	Purification Factor
Starting Filtrate	1
After Cation exchanger pH 4.9	1.07
After Cation exchanger pH 3.8	1.2
After Anion exchanger	1.46

The final (anion exchanged) product containing 10mg protein/ml was concentrated ten-fold by ultrafiltration using an Amicon Stirred Cell (2L module) with a Kalle E35 membrane at 3 bar.

The final concentration for purified phytase reached 280-300g/l (28-30%). With a specific activity of 100 FTU/mg protein, this results in a phytase activity of 28,000-30,000 FTU/g.

EXAMPLE 5

High Activity Phytase Stability Tests

To demonstrate that a higher enzyme concentration (in granules made using the high activity phytase liquid) gives a higher pelleting stability, granulates with an increasing enzyme concentration were made and the pelleting stability of these samples were tested.

Comparative Sample A: Preparation of a corn starch-based low active enzyme granulate by mixing, kneading, extrusion, spheronisation and drying.

A mixture was prepared by mixing and kneading 73% (w/w) corn starch and low concentration 4% (w/w) phytase Ultra Filtrate and 23% (w/w) water. This mixture was extruded using a Nica E-220 basket extruder to obtain a wet extrudate which was spheronised in a Fuji Paudal Marumeriser™ for 2 minutes to obtain round particles of an average diameter of 600µm. These particles were subsequently dried in a Glatt GPCG 1.1 fluid bed dryer. The final activity of the granulate was 610 FTU/g.

Comparative Sample B: Preparation of a corn starch-based middle active enzyme granulate by mixing, kneading, extrusion, spheronisation and drying.

A mixture was prepared by mixing and kneading 70% (w/w) corn starch and 17% (w/w) phytase Ultra Filtrate and 13% (w/w) water. This mixture was extruded

using a Nica E-220 basket extruder to obtain a wet extrudate which was spheronised in a Fuji Paudal Marumeriser for 2 minutes to obtain round particles of an average diameter of 600µm. These particles were subsequently dried in a Glatt GPCG 1.1 fluid bed dryer. The final activity of the granulate was 4170 FTU/g.

Sample C

Preparation of a corn starch-based high active enzyme granulate by mixing, kneading, extrusion, spheronisation and drying.

A mixture was prepared by mixing and kneading 67% (w/w) corn starch and 30% (w/w) of the phytase Ultra Filtrate prepared in Example 4 (but diluted to 18,400 FTU/g) and 3% (w/w) water. This mixture was extruded using a Nica E-220 basket extruder to obtain a wet extrudate which was spheronised in a Fuji Paudal Marumeriser for 2 minutes to obtain round particles of an average diameter of 600µm. These particles were subsequently dried in a Glatt GPCG 1.1 fluid bed dryer. The final activity of the granulate was 6830 FTU/g.

Comparison of the pelleting stabilities

The different enzyme granulates were subsequently placed in a pelleting trial and their pelleting stability compared. The pelleting trial consists of mixing the enzyme granulates with a feed premix at respectively 1500, 320 and 200ppm. These mixtures were pre-treated by steam injection to give a temperature rise to 75°C, after which the mixtures were pelleted in a pelleting machine to obtain the feed pellets at a temperature of 82°C, which were subsequently dried. This type of process is typical for the feed industry to obtain feed pellets.

Table 2 summarises the results of the pelleting trials. It is apparent that the two granules with the highest enzyme concentration had much higher pelleting stability.

Table 2: Results of the pelleting tests

Sample number	Granulate activity in FTU/g	Temp. of meal (°C)	Temp. Pellets (°C)	Enzyme yield after pelleting (%)
Comp. (A)	610	75	82	<17
Comp. (B)	4,170	75	82	37
(C)	6,830	75	82	48

EXAMPLE 6

Preparation of a potato starch-based enzyme granulate containing soy oil and $MgSO_4$ additions by mixing, kneading, pelleting and drying

In a mixer/kneader 30 kg of potato starch was added and 2.5 kg of Soy oil was mixed in. Subsequently a phytase ultra-filtrate derived from *Aspergillus* (16,840 FTU/g) was added containing $MgSO_4.7H_20$ (3.5 kg of $MgSO_4.7H_20$ was dissolved in 14 kg of ultra-filtrate). The product was mixed thoroughly in the kneader, then extruded and dried in a fluid bed drier as in Example 1. This resulted in a product of 5870 FTU/g.

EXAMPLE 7

Preparation of a rice starch-based enzyme granulate by mixing, kneading, extrusion, spheronisation and drying

A mixture was prepared by mixing and kneading 62% (w/w) rice starch and 38% (w/w) of the same phytase ultra-filtrate used in Example 6. This mixture was extruded using the Fuji Paudal basket extruder to obtain a wet extrudate which was then spheronised in the MARUMERISER™ for one minute to obtain round particles of an average diameter of 785 μm. These particles were subsequently dried in a fluid bed drier as in Example 1. The final activity of the granulate was 7280 FTU/g.

EXAMPLE 8

Preparation of a corn starch-based enzyme granulate containing an HPMC addition by mixing, kneading, extrusion, spheronisation and drying

An enzyme preparation was obtained by kneading a mixture of 54% (w/w) of corn starch, 5% of HPMC (hydroxy-propyl-methyl-cellulose) and 41% (w/w) of the phytase ultra-filtrate used in Example 6. This mixture was extruded using the Fuji Paudal basket extruder to obtain a wet extrudate which was spheronised in the MARUMERISER™ for one minute to obtain round particles of an average diameter of 780 μm. These were subsequently dried in a fluid bed drier for 20 minutes at 40°C bed temperature, and 75°C inlet temperature. The thus obtained dry enzyme granulate had an activity of 8470 FTU/g.

EXAMPLE 9

Preparation of a corn starch-based enzyme granulate containing an HEC addition by mixing, kneading, extrusion, spheronisation and drying

An enzyme preparation was obtained by mixing and kneading 54% (w/w) of corn starch, 5% (w/w) of HEC (hydroxy-ethyl-cellulose) with 41% (w/w) of the same phytase ultra-filtrate used in Example 6. This mixture was extruded using the Fuji Paudal basket extruder to obtain a wet extrudate which was spheronised in the MARUMERISER™ for one minute to obtain round particles of an average diameter of 780 μm. These were subsequently dried in a fluid bed drier for 20 minutes at 40°C bed temperature, and 75°C inlet temperature. The thus obtained dry enzyme granulates had an activity of 8410 FTU/g.

EXAMPLE 10

An ultrafiltrate of 18,000 FTU/g was employed, derived from the ultrafiltrate from Example 4, and diluted.

Samples

The activity of the 3 samples prepared were 610 (A, Comparative); 4170 (B, Comparative) and 6830 (C) FTU/g. This gave three feeds of activity 1.153, 1.685 and 1.745 FTU/g feed, respectively.

The first sample, 150g was mixed with 20 kg feed as described below. After this the premix was mixed with 80kg feed and divided in two parts to become feed for two trials at two different temperatures. The second sample was 153.6g in 20kg feed. This 20,153.6g sample was divided in two equal portions of 10.076kg. Each portion was then mixed with 230kg feed to get the meal for the tests.

For the third trial 96g of granulate was mixed with 20kg feed and divided in two portions of 10.048g. Each portion was then mixed with 230kg feed to get the meal for the tests. The pelleting speed was 600kg/h. The feed mixture is set out in Table 3.

Table 3: Feed Mixture

Corn	20.00%
Wheat	30.00%
Soybeans (heated)	10.00%
Soy (coarse meal 46.7/3.7)	18.20%
Tapioca (65 % starch)	6.97%
Animal meal (56.5/10.9)	4.00%
Fish meal (70.6 % re)	1.00%
Feather meal, hydr.	1.00%
Soy oil/ maize oil	1.30%
Animal fat	4.00%
Vit./Min.premix (maize)	1.00%
Calcium carbonate	0.85%
Mono-calcium phosphate	1.05%
Salt	0.26%
L-Lysine HCl	0.16%
DL-Methionine	0.21%

The three mixtures were then pelleted. The feed was fed into a conditioner where direct steam was added to the meal. The temperature rose to 75°C. Subsequently the meal exited the pelletiser where it was pushed through a die plate with 5 mm holes and 65 mm thick. The temperature of the feed at this point rose another 4°C to 79°C.

The activity of the three feeds was 10.11(A); 10.04(B) and 9.81(C).

The results of this test for residual activity were: 63(A); 66(B) and 72%(C) respectively for the original 610; 4170; 6830 and FTU/g samples. This shows that even with similar activities (B and C) the highest activity formulation (C; 6830 FTU/g, of the invention) gave a much higher pelleting stability. This was 6% higher than for (comparative) Sample B, remarkable as only a 3% increase was observed (from A to B) with a very large increase in activity (610 to 4170 FTU/g).

All the references cited herein, including patents, patent applications, and publications, are hereby incorporated in their entireties by reference.